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(54) NON-ANTIGENIC BRANCHED POLYMER CONJUGATES

NICHT-ANTIGENE VERZWEIGTE POLYMER-KONJUGATE

CONJUGUES POLYMERES RAMIFIES NON ANTIGENIQUES

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Description

Field of the Invention

[0001] The present invention relates to branched polymers which are useful in extending the in vivo circulating life of biologically active materials. The invention also relates to conjugates made with the polymers.

[0002] Some of the initial concepts of coupling peptides or polypeptides to poly(ethylene glycol) PEG and similar water-soluble poly(alkylene oxides) are disclosed in U.S. Patent No. 4,179,337, the disclosure of which is incorporated herein by reference. Polypeptides modified with these polymers exhibit reduced immunogenicity/antigenicity and circulate in the bloodstream longer than unmodified versions.

[0003] To conjugate poly(alkylene oxides), one of the hydroxyl end-groups is converted into a reactive functional group. This process is frequently referred to as "activation" and the product is called an "activated poly (alkylene oxide) ". Other substantially non-antigenic polymers are similarly "activated" or functionalized.

[0004] The activated polymers are reacted with a therapeutic agent having nucleophilic functional groups that serve as attachment sites. One nucleophilic functional group commonly used as an attachment site is the ε-amino groups of lysines. Free carboxylic acid groups, suitably activated carbonyl groups, oxidized carbohydrate moieties and mercapto groups have also been used as attachment sites.

[0005] Insulin and hemoglobin were among the first therapeutic agents conjugated. These relatively large polypeptides contain several free ε-amino attachment sites. A sufficient number of polymers could be attached to reduce immunogenicity and increase the circulating life without significant loss of biologic activity.

[0006] Excessive polymer conjugation and/or conjugation involving a therapeutic moiety's active site where groups associated with bioactivity are found, however, often result in loss of activity and thus therapeutic usefulness. This is often the case with lower molecular weight peptides which have few attachment sites not associated with bioactivity. Many non-peptide therapeutics also lack a sufficient number of attachment sites to obtain the benefit of polymer modification.

[0007] One suggestion for overcoming the problems discussed above is to use longer, higher molecular weight polymers. These materials, however, are difficult to prepare and expensive to use. Further, they provide little improvement over more readily available polymers.

[0008] Another alternative suggested is to attach two strands of polymer via a triazine ring to amino groups of a protein. See, for example, Enzyme, 26, 49-53 (1981) and Proc. Soc. Exper. Biol. Med., 188, 364-9 (1988).

[0009] Research in this area has continued. Triazine is a toxic substance which is difficult to reduce to acceptable levels after conjugation. In addition, triazine is a planar group and can only be double-polymer substituted. The planar structure rigidly locks the two polymer chains in place. This limits the benefits of polymer conjugation to about the same as that obtained by increasing polymer chain length. Thus, non-triazine-based activated polymers would offer substantial benefits to the art. The present invention addresses this need.

[0010] US patent 5,183,660 relates to polyethylene glycol derivatives useful for modifying peptides. The derivatives are di-substituted benzene derivatives as an alternative linking moiety to address the shortcomings associated with triazine-based linkers.

[0011] US patent 4,766;106 discloses modification of biologically active lipophilic proteins to render them soluble at physiological pH. The proteins are water-insoluble proteins like interleukin-2, interferon-beta and immunotoxins. To render them soluble in water at physiological pH, they are modified with polyethylene glycol, monomethyl polyethylene glycol or polyoxyethylated glycerol. These are coupled to the protein via an amide linkage formed from the 4-hydroxy-3-nitrobenzene sulfonate ester or the N-hydroxysuccinimide ester.

[0012] WO 93/24476 discloses a modification of taxol to render it soluble in water. Taxol is covalently bound to water-soluble polyethylene glycols, such as linear, branched or star polyethylene glycols and branched copolymers of polyethylene glycols with other functional monomers, such as acrylic acid.

[0013] WO 90/13540 discloses the N-succinimide carbonate derivative of poly(ethylene glycol) for modification of proteins. There is no disclosure of branched polymers.

[0014] WO 88/08992 relates to a chemically or biologically sensing optical fiber. Monoclonal antibodies are used as sensing material. Branched polyethylene oxides having a molecular weight up to 3000 are used to couple the antibodies to the optical fibers. The polymers may be synthesized by polymerization of ethylene oxide in the presence of some glycidol anion. The branched polyethylene oxides contain two or three primary alcohol moieties and do not comprise an aliphatic linking moiety as the polymers of the invention.

SUMMARY OF THE INVENTION

[0015] In one aspect of the invention, there are provided branched, substantially non-antigenic polymers comprising the formula:

$$(R)_nL\text{-}A \qquad (I)$$

wherein

(R) is a water-soluble, substantially non-antigenic polymer containing a terminal C_1 - C_4 group ;
(n) = 2 or 3;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each *(R)* ; and
(A) is a functional group capable of bonding with a biologically active nucleophile or a moiety capable of being functionalized to react with said nucleophile.

[0016] In particularly preferred aspects of the invention, (R) includes a poly(alkylene oxide) PAO such as a poly (ethylene glycol) PEG.

[0017] These umbrella-like branched polymers of the present invention (U-PAO's or U-PEG's) react with biologically active nucleophiles to form conjugates. The point of polymer attachment depends upon the functional group *(A)*. For example, *(A)* can be a succinimidyl succinate or carbonate and react with epsilon amino lysines. The branched polymers can also be activated to link with any primary or secondary amino group, mercapto group, carboxylic acid group, reactive carbonyl group or the like found on biologically-active materials. Other groups are apparent to those of ordinary skill in the art.

[0018] Other aspects of the invention include conjugates containing biologically-active materials and one or more of the branched polymers described above as well as methods of their preparation. The biologically active materials include proteins, peptides, enzymes, medicinal chemicals or organic moieties whether synthesized or isolated from nature. The methods include contacting a biologically active material containing a nucleophile capable of undergoing a substitution reaction with a branched polymer described above under conditions sufficient to effect attachment while maintaining at least a portion of the biological activity.

[0019] It is possible to use the invention for treating various maladies and conditions. A mammal in need of treatment may be administered an effective amount of a conjugate containing a biologically-active material such as a protein, enzyme or organic moiety and a branched polymer of the present invention.

[0020] One of the chief advantages of the present invention is that the branching of the polymers imparts an umbrella-like three-dimensional protective covering to the materials they are conjugated with. This contrasts with the string-like structure of conventional polymer conjugates. Moreover, the branching of the polymer chains from a common root allows dynamic, non-planar action in vivo. Thus, the branched polymers offer substantial benefits over straight-chained polymers of equivalent molecular weight.

[0021] A second advantage of the branched polymers is that they provide the benefits associated with attaching several strands of polymers to a bioeffecting material but require substantially fewer conjugation sites. The advantages of the branched polymers are particularly dramatic for therapeutic agents having few available attachment sites. All the desired properties of polymer conjugation are realized and loss of bioactivity is minimized.

DETAILED DESCRIPTION OF THE INVENTION

1. POLYMER SUBSTITUENTS AND FORMULA I DEFINED

[0022] The activated branched polymers of the present invention are preferably prepared from poly(alkylene oxides) (PAO's) that are water soluble at room temperatures. Within this group are alpha-substituted polyalkylene oxide derivatives such as methoxypoly (ethylene glycols) (mPEG) or other suitable alkyl substituted PAO derivatives such as those containing mono or bis terminal C_1 - C_4 groups. Straight-chained non-antigenic polymers such as monomethyl PEG homopolymers are preferred. Alternative polyalkylene oxides such as other poly(ethylene glycol) homopolymers, other alkyl-poly(ethylene oxide) block copolymers, and copolymers of block copolymers of poly(alkylene oxides) are also useful.

[0023] The polymers of the present invention are represented by Formula (I):

$$(R)_nL\text{-}A \qquad (I)$$

wherein:

(R) includes a water-soluble, substantially non-antigenic polymer;
(n) = 2 or 3;
(L) is an aliphatic linking moiety covalently linked to each *(R)*; and
(A) represents an activating functional group capable of undergoing nucleophilic substitution.

[0024] Each *(R)* can be a water-soluble, substantially non-antigenic polymer chain. When the polymer chains are PEG or mPEG, it is preferred that each chain have a molecular weight of between about 200 and about 20,000 daltons and preferably between about 1,000 and about 10,000 daltons. Molecular weights of about 5,000 daltons are most preferred.

[0025] Alternative polymeric substances include materials such as dextrans, polyvinyl pyrrolidones, polyacrylamides or other similar non-immunogenic polymers. Such polymers are also capable of being functionalized or activated for inclusion in the invention. The foregoing is merely illustrative and not intended to restrict the type of non-antigenic polymers suitable for use herein.

[0026] In another embodiment of the invention, *(R)* is a branched polymer for secondary and tertiary branching from a bioactive material. Bifunctional and heterobifunctional active polymer esters can also be used. The polymers of the present invention can also be copolymerized with bifunctional materials such as poly(alkylene glycol) diamines to form interpenetrating polymer networks suitable for use in permeable contact lenses, wound dressings, drug delivery devices and the like. The stearic limitations and water solubility of such branching will be readily recognized by one of ordinary skill in the art. Preferably, however, the molecular weight of multiply branched polymers should not exceed 80,000 daltons.

[0027] As shown in Formula I, 2 or 3 polymer chains, designated *(R)* herein, are joined to the aliphatic linking moiety *(L)*. Suitable aliphatics include substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives. The linking moieties are preferably non-planar, so that the polymer chains are not rigidly fixed. The linking moiety *(L)* is also the means for attaching the multiple polymer chains or "branches" to *(A)*, the moiety through which the polymer attaches to bio-effecting materials.

[0028] *(L)* preferably includes a multiple-functionalized alkyl group containing up to 18, and more preferably between 1-10 carbon atoms. A heteroatom such as nitrogen, oxygen or sulfur may be included within the alkyl chain. The alkyl chain may also be branched at a carbon or nitrogen atom.

[0029] *(L)* and each *(R)* are preferably joined by a reaction between nucleophilic functional groups on both *(R)* and *(L)*. Each *(R)* is suitably functionalized to undergo nucleophilic substitution and bond with *(L)*. Such functionalization of polymers is readily apparent to those of ordinary skill in the art.

[0030] A wide variety of linkages are contemplated between *(R)* and *(L)*. Urethane (carbamate) linkages are preferred. The bond can be formed, for example, by reacting an amino group such as 1,3-diamino-2-propanol with methoxypolyethylene glycol succinimidyl carbonate described in U.S. Patent No. 5,122,614, the disclosure of which is incorporated herein by reference. Amide linkages can be formed by reacting an amino-terminated non-antigenic polymer such as methoxypolyethylene glycol-amine (mPEG amine) with an acyl chloride functional group.

[0031] Examples of other linkages between *(R)* and *(L)* include ether, amine, urea, and thio and thiol analogs thereof, as well as the thio and thiol analogs of the above-discussed urethane and amide linkages. The linkages are formed by methods well understood by those of ordinary skill in the art. Other suitable linkages and their formation can be determined by reference to the above-cited U.S. Patent No. 4,179,337.

[0032] The moiety *(A)* of Formula I represents groups that "activate" the branched polymers of the present invention for conjugation with biologically active materials.

(A) can be a moiety selected from:

I. Functional groups capable of reacting with an amino group such as:

a) carbonates such as the p-nitrophenyl, or succinimidyl;
b) carbonyl imidazole;
c) azlactones;
d) cyclic imide thiones; or
e) isocyanates or isothiocyanates.

II. Functional groups capable of reacting with carboxylic acid groups and reactive carbonyl groups such as:

a) primary amines; or
b) hydrazine and hydrazide functional groups such as the acyl hydrazides, carbazates, semicarbamates, thiocarbazates, etc.

III. Functional groups capable of reacting with mercapto or sulfhydryl groups such as phenyl glyoxals; see, for example, U.S. Patent No. 5,093,531, the disclosure of which is hereby incorporated by reference.

IV. Other nucleophiles capable of reacting with an electrophilic center. A non-limiting list includes, for example, hydroxyl, amino, carboxyl, thiol groups, active methylene and the like.

[0033] The moiety *(A)* can also include a spacer moiety located proximal to the aliphatic linking moiety, (*L*). The spacer moiety may be a heteroalkyl, alkoxy, alkyl containing up to 18 carbon atoms or even an additional polymer chain. The spacer moieties can added using standard synthesis techniques. It is to be understood that those moieties selected for *(A)* can also react with other moieties besides biologically active nucleophiles.

2. SYNTHESIS OF BRANCHED POLYMERS

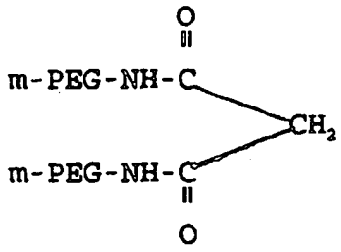
[0034] The branched polymers (generally, U-PAO's or U-PEG's) are formed using conventional reaction techniques. For each polymer chain (*R*) attached, the linking compound *(L)* has a number of nucleophilic functional groups which correspond to *(n)*, (i.e. 2 or 3). In one aspect, a succinimidyl carbonate active ester of the branched polymer is prepared by contacting a branched polymer subunit $(R)_nL$, prepared as described above, with p-nitrophenyl chloroformate and thereafter with N-hydroxysuccinimide to form a succinimidyl carbonate. Alternatively, the hydroxy moiety can be reacted with bis-succinimidyl carbonate directly. The polymer subunit $(R)_nL$ will include hydroxyl, amino, carboxyl and thiol groups, and the like, as well as amino or methylene hydrogens so that it can be attached to (*A*).

[0035] The branched polymers can also be formed by reacting aliphatic linking compounds substituted with nucleophilic functional groups such as di- or tri-amino, mercapto alcohols or alkyl triols with an activated or functionalized polymer chain such as SC-PEG, PEG-NCO, PEG-NCS, SS-PEG, PEG-acids and acid derivatives. Such methods are preferred because functionalized polymer chains and suitable aliphatic linking groups are either commercially available or readily synthesized.

[0036] Other aspects of synthesis include reacting a polymer functionalized with a nucleophilic moiety such as PEG-alcohol, PEG-amine or PEG-mercaptan with bifunctional molecules such as malonic acid derivatives or glyoxalic acid derivatives.

[0037] For example, two moles of methoxy-poly(ethylene glycol) amine can be reacted with a substituted or unsubstituted malonyl chloride to form a compound of Formula (II) :

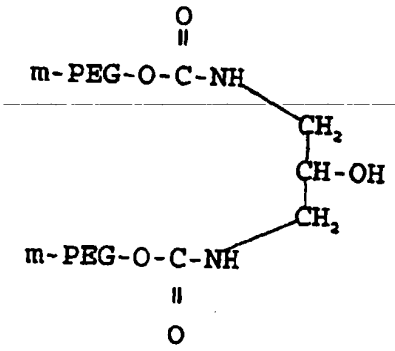
(II)



Reaction with strong base converts the methylene linker into an anion that can be further functionalized.

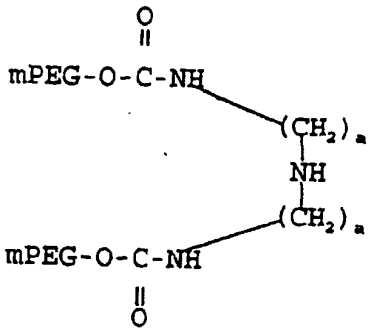
[0038] Likewise, two moles of methoxy-poly (ethylene glycol) succinimidyl carbonate may be reacted with a 1,3 diamino 2-propanol to form a compound of Formula (III):

(III)



[0039] Similarly, two moles of mPEG can be reacted with a triamine such as diethylenetriamine to form a compound having the structure of Formula (IV):

(IV)



a=1-5

Branched polymer (III) can then be activated by first functionalizing with compounds capable of activating the hydroxyl group such as p-nitrophenyl chloroformate to form a reactive p-nitrophenyl carbonate. The resulting p-nitrophenyl carbonate polymer can be directly reacted with a biologically active nucleophile.

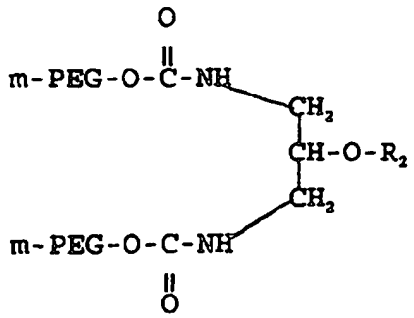
[0040] The p-nitrophenyl carbonate polymer can also serve as an intermediate. It can be reacted with a large excess of N-hydroxysuccinimideto form a succinimidyl carbonate-activated branched polymer. Other routes to succinimidyl carbonates are available and contemplated for use herein. Alternatively, a p-nitrophenyl carbonate polymer intermediate can be reacted with anhydrous hydrazine to form a carbazate branched polymer.

[0041] Branched polymer (IV) can be activated by reacting (IV) with a hydroxy acid such as lactic acid to form the hydroxy amide. Thereafter, the hydroxy amide is functionalized in the same manner discussed above for (III).

[0042] As will be readily appreciated, numerous variations and combinations of the reaction between the functionalized polymer chains and aliphatic linking compound can be utilized to form the compounds of the present invention. The foregoing reactions were disclosed to illustrate the present invention.

[0043] Branched polymers corresponding to Formula (II), Formula (III), and the like, can also be extended with a spacer moiety, designated herein as R_2, between the aliphatic linking moiety and the group capable of undergoing nucleophilic substitution. For example, the polymer of Formula (III) with a spacer moiety is represented by Formula (V):

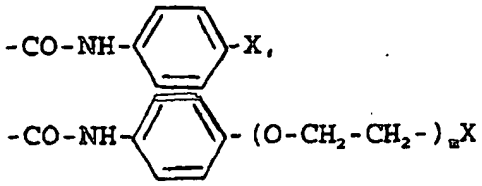
(V)



Spacer moieties represented by (R_2) include but are not limited to:

$$-CO-NH-(CH_2-)_mX$$

$$-CO-NH-(CH_2-CH_2-O-)_mX$$



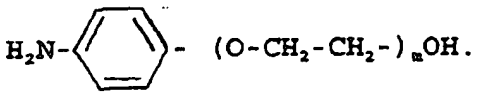
and the like, where *(m)* is an integer between 1 and 18 inclusive and *(X)* = H, OH, NH_2, COOH. Depending upon the circumstances, an -H of an -OH group is attached to the end of the spacer moiety to form the terminal hydroxyl group.

[0044] Synthesis of compounds corresponding to (V) include reacting the p-nitrophenyl carbonate or N-succinimidyl carbonate active esters of Formula (III) compounds with reagents such as

$$H_2N-(CH_2-)_mOH,$$

$$H_2N-(CH_2-CH_2-O-)_mH,$$

aminophenols, or



[0045] The attachment of spacer moieties to a branched polymer is described with reference to the polymer of Formula (II) for purposes of illustration, not limitation. Similar products would be obtained with any of the branched polymers disclosed by the present invention. For example, spacer moieties (R_2) can be joined to linker moieties (*L*) substituted with groups other than hydroxyl groups. When the hydroxyl group is replaced by an amino group, or when the carbon substituted with hydroxyl groups is replaced by a secondary amine, (*L*) can be reacted with suitable reagents such as substituted isocyanates or isothiocyanates and the like. Like the aliphatic linking moieties described above, the terminal groups of the spacer moieties can be similarly functionalized to react with nucleophiles.

[0046] After synthesis, the activated branched polymers can be purified by conventional methods and reacted with biologically active materials containing nucleophiles capable of bonding with the polymer while maintaining at least some of the activity associated with the material in unmodified form.

3. BIOLOGICALLY ACTIVE MATERIALS SUITABLE FOR CONJUGATION

[0047] The nucleophiles conjugated with the branched polymers are described as "biologically active". The term, however, is not limited to physiological or pharmacological activities. For example, some nucleophile conjugates such as those containing enzymes, are able to catalyze reactions in organic solvents. Likewise, some inventive polymer conjugates containing proteins such as concanavalin A, immunoglobulin and the like are also useful as laboratory diagnostics. A key feature of all of the conjugates is that at least some portion of the activity associated with the unmodified bio-active material is maintained.

[0048] The conjugates are biologically active and have numerous therapeutic applications. Mammals in need of treatment which includes a biologically active material can be treated by administering an effective amount of a polymer conjugate containing the desired bioactive material. For example, mammals in need of enzyme replacement therapy or blood factors can be given branched polymer conjugates containing the desired material.

[0049] Biologically active nucleophiles of interest of the present invention include, but are not limited to, proteins, peptides, polypeptides, enzymes, organic molecules of natural and synthetic origin such as medicinal chemicals and the like.

[0050] Enzymes of interest include carbohydrate-specific enzymes, proteolytic enzymes, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Without being limited to particular enzymes, examples of enzymes of interest include asparaginase, arginase, arginine deaminase, adenosine deaminase, superoxide dismutase, endotoxinases, catalases, chymotrypsin, lipases, uricases, adenosine diphosphatase, tyrosinases and bilirubin oxidase. Carbohydrate-specific enzymes of interest include glucose oxidases, glucodases, galactosidases, glucocerebrosidases, glucouronidases, etc.

[0051] Proteins, polypeptides and peptides of interest include, but are not limited to, hemoglobin, serum proteins such as blood factors including Factors VII, VIII, and IX; immunoglobulins, cytokines such as interleukins, α-, β- and γ-interferons, colony stimulating factors including granulocyte colony stimulating factors, platelet derived growth factors and phospholipase-activating protein (PLAP). Other proteins of general biological or therapeutic interest include insulin, plant proteins such as lectins and ricins, tumor necrosis factors and related alleles, growth factors such as tissue growth factors, such as TGFα's or TGFβ's and epidermal growth factors, hormones, somatomedins,' erythropoietin, pigmentary hormones, hypothalamic releasing factors, antidiuretic hormones, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, and the like. Immunoglobulins of interest include IgG, IgE, IgM, IgA, IgD and fragments thereof.

[0052] Some proteins such as the interleukins, interferons and colony stimulating factors also exist in non-glycosylated form, usually as a result of using recombinant techniques. The non-glycosylated versions are also among the biologically active nucleophiles of the present invention.

[0053] The biologically active nucleophiles of the present invention also include any portion of a polypeptide demonstrating in vivo bioactivity. This includes amino acid sequences, antisense moieties and the like, antibody fragments, single chain binding proteins, see, for example U.S. Patent No. 4,946,778, disclosure of which is incorporated herein by reference, binding molecules including fusions of antibodies or fragments, polyclonal antibodies, monoclonal antibodies, catalytic antibodies, nucleotides and oligonucleotides.

[0054] The proteins or portions thereof can be prepared or isolated by using techniques known to those of ordinary skill in the art such as tissue culture, extraction from animal sources, or by recombinant DNA methodologies. Transgenic sources of the proteins, polypeptides, amino acid sequences and the like are also contemplated. Such materials are obtained form transgenic animals, i.e., mice, pigs, cows, etc., wherein the proteins expressed in milk, blood or tissues. Transgenic insects and baculovirus expression systems are also contemplated as sources. Moreover, mutant versions of proteins, such as mutant TNF's and/or mutant interferons are also within the scope of the invention.

[0055] Other proteins of interest are allergen proteins such as ragweed, Antigen E, honeybee venom, mite allergen, and the like.

[0056] Useful biologically active nucleophiles are not limited to proteins and peptides. Essentially any biologically-active compound is included within the scope of the present invention. The present invention is particularly well-suited for compounds which have few or even a single nucleophilic attachment site for polymer conjugation such as medicinal chemicals whether isolated from nature or synthesized. Chemotherapeutic molecules such as pharmaceutical chemicals i.e. anti-tumor agents, anti-neoplastics, anti-infectives, anti-anxiety agents, gastrointestinal agents, central nervous system-activating agents, analgesics, fertility or contraceptive agents, anti-inflammatory agents, steroidal agents, anti-urecemic agents, cardiovascular agents, vasodilating agents, vasoconstricting agents and the like.

[0057] The foregoing is illustrative of the biologically active nucleophiles which are suitable for conjugation with the polymers of the invention. It is to be understood that those biologically active materials not specifically mentioned but having suitable nucleophilic groups are also intended and are within the scope of the present invention.

4. SYNTHESIS OF BIOLOGICALLY ACTIVE CONJUGATES

[0058] One or more of the activated branched polymers can be attached to a biologically active nucleophile by standard chemical reactions. The conjugate is represented by the formula:

$$(VI) \qquad [(R)_n L\text{-}A^1]_z\text{-} (\text{nucleophile})$$

wherein *(R)* is a water-soluble substantially non-antigenic polymer; n = 2 or 3; (*L*) is an aliphatic linking moiety; (A^1) represents a linkage between *(L)* and the nucleophile and *(z)* is an integer ≥ 1 representing the number of polymers conjugated to the biologically active nucleophile. The upper limit for (z) will be determined by the number of available nucleophilic attachment sites and the degree of polymer attachment sought by the artisan. The degree of conjugation can be modified by varying the reaction stoichiometry using well-known techniques. More than one polymer conjugated to the nucleophile can be obtained by reacting a stoichiometric excess of the activated polymer with the nucleophile.

[0059] The biologically active nucleophiles can be reacted with the activated branched polymers in an aqueous reaction medium which can be buffered, depending upon the pH requirements of the nucleophile. The optimum pH for the reaction is generally between about 6.5 and about 8.0 and preferably about 7.4 for proteinaceous/polypeptide materials. Organic/chemotherapeutic moieties can be reacted in non-aqueous systems. The optimum reaction conditions for the nucleophile's stability, reaction efficiency, etc. is within level of ordinary skill in the art. The preferred temperature range is between 4°C and 37°C. The temperature of the reaction medium cannot exceed the temperature at which the nucleophile may denature or decompose. It is preferred that the nucleophile be reacted with an excess of the activated branched polymer. Following the reaction, the conjugate is recovered and purified such as by diafiltration, column chromatography, combinations thereof, or the like.

[0060] It can be readily appreciated that the activated branched non-antigenic polymers of the present invention are a new and useful tool in the conjugation of biologically active materials, especially when they lack a sufficient number of suitable polymer attachment sites.

EXAMPLES

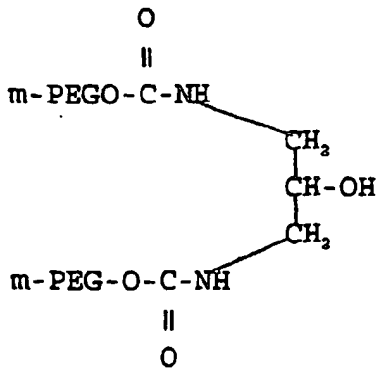
[0061] The following non-limiting examples illustrate certain aspects of the invention. All parts and percentages are by weight unless otherwise noted and all temperatures are in degrees Celsius.

MATERIALS

[0062] Methoxypoly(ethylene glycol) (m-PEG) was obtained from Union Carbide. The solvents were obtained from Aldrich Chemical of Milwaukee, Wisconsin. The methoxy-poly(ethylene glycol)-N-succinimidyl carbonate (SC-PEG) was prepared as described in U.S. Patent No. 5,122,614, using m-PEG having a molecular weight of about 5,000. Each of the products prepared in Examples 1 - 9 were confirmed structurally by carbon - 13 NMR.

EXAMPLE 1 - **U-PEG-OH**

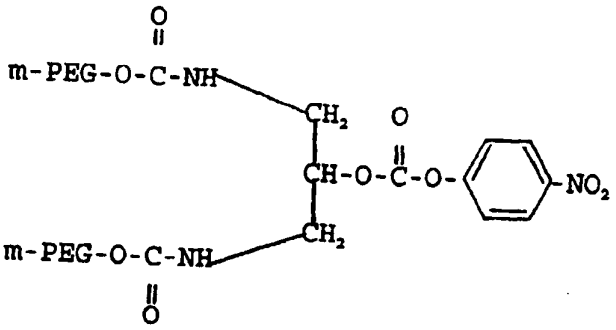
[0063]



This branched polymer was prepared by adding 100 mg (1.1 mmol) of 1, 3-diamino-2-propanol to a solution of 10.0 g (2 mmol) of SC-PEG in 50 mL of methylene chloride. The mixture was stirred for 18 hours at room temperature then filtered. Excess solvent was removed by distillation in vacuo. The residue was recrystallized from 2-propanol to yield 7.1 g of product (70% yield).

EXAMPLE 2 - **U-PNP-PEG**

[0064]

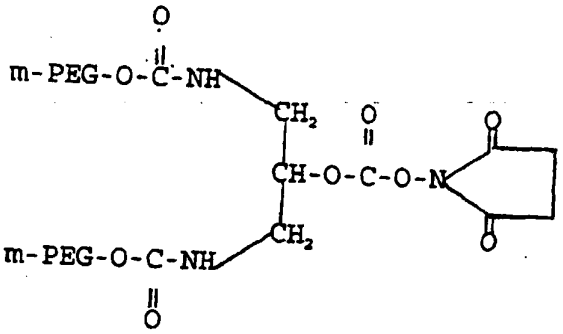


[0065] The compound of Example 1 was activated with p-nitrophenyl chloroformate. First, 5.0g (0.5 mmol) of U-PEG was azeotropically dried by refluxing in 75 mL of toluene for 2 hours, resulting in the removal of 25 mL of solvent/water. The reaction mixture was cooled to 30°C, followed by the addition of 120 mg (0.6 mmol) of p-nitrophenyl chloroformate and 50 mg (0.6 mmol) of pyridine. The resulting mixture was stirred for two hours at 45°C, followed by stirring overnight at room temperature.

[0066] The reaction mixture was then filtered through CELITE™, followed by removal of the solvent from the filtrate by distillation in vacuo. The residue was recrystallized from.2-propanol to yield 4.2 g (81% yield) of the product.

EXAMPLE 3 - **US-PEG**

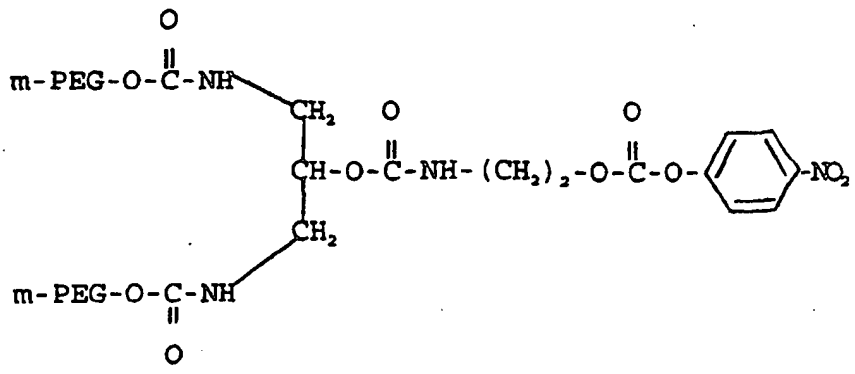
[0067]



[0068] In this example, the U-PNP PEG of Example 2 was reacted with N-hydroxysuccinimide to form the succinimidyl carbonate ester of U-PEG. A solution containing 5.0 g (0.5 mmol) of the U-PNP PEG, 0.6 g (5 mmol) of N-hydroxy-succinimide and 0.13 g (1 mmol) of diisopropylethylamine in 40 ml of methylene chloride was refluxed for 18 hours. The solvent was then removed by distillation in vacuo, and the residue was recrystallized from 2-propanol to yield 4.2 g of the succinimidyl carbonate ester (82% yield).

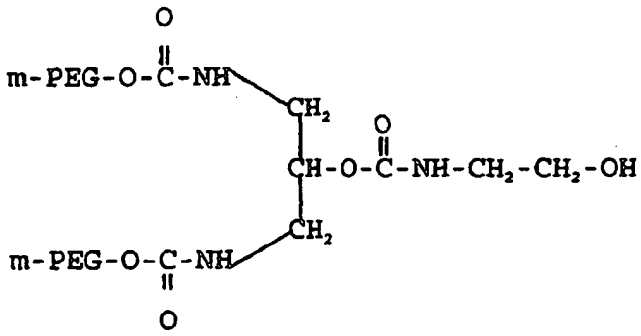
EXAMPLE 4 - **NU-PNP-PEG**

[0069]



[0070] This branched polymer above was prepared by reacting U-PNP PEG (Ex. 2) with ethanolamine followed by p-nitrophenyl chloroformate.

[0071] A solution containing 5.0 g (0.5 mmol) of U-PNP PEG in 40 mL of methylene chloride was combined with 60 mg (1 mmol) of ethanolamine and stirred overnight at room temperature. Thereafter, the solvent was removed by distillation in vacuo. The residue was recrystallized from 2-propanol to yield 4.3 g of the intermediate (84% yield) shown below:

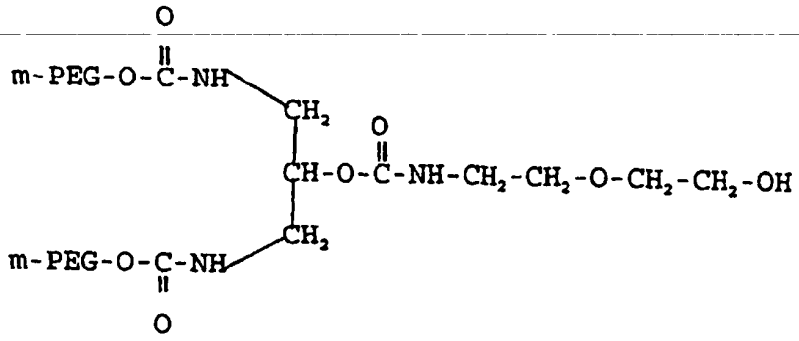


[0072] The NU-PEG-OH was prepared by reacting the above intermediate with p-nitrophenyl chloroformate. The intermediate was azeotropically dried by refluxing, 2.0 g (0.2 mmol) in 40 mL toluene for two hours, with the removal of 25 mL of solvent/water. The reaction mixture was cooled, followed by the addition of 0.3 mmol p-nitrophenyl chloroformate and 0.3 mmol pyridine, according to the procedure of Example 2. The resulting mixture was stirred for two hours at 45°C, followed by stirring overnight at room temperature.

[0073] The NU-PEG-OH was also recovered by the procedure in Example 2 to yield 1.5 g (71% yield).

EXAMPLE 5 - **XU-PEG-OH**

[0074]



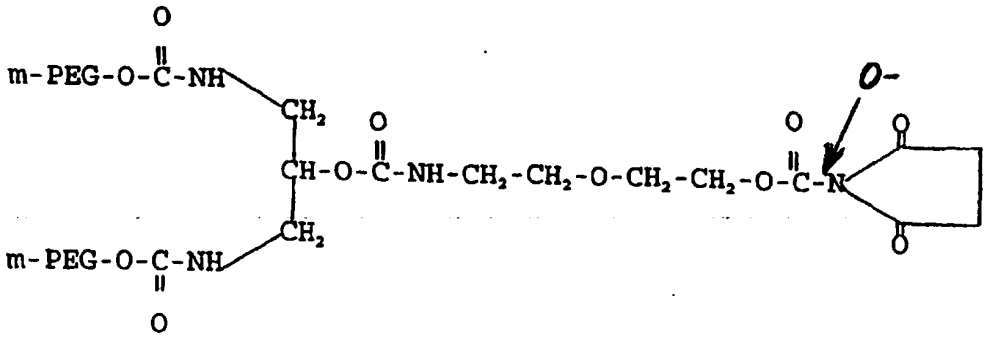
[0075] This branched polymer was prepared by reacting the U-PNP PEG of Example 2 with 2-(2-aminoethoxy) ethanol according to the procedure described in Example 4, (i.e., the amino alcohol was reacted with the p-nitrophenyl carbonate). The recrystallized product yield was 86%.

EXAMPLE 6 - **XU-PNP-PEG**

[0076] The compound of Example 5 was functionalized with p-nitrophenyl carbonate as in Examples 2 and 4. The recrystallized product yield was 83%.

EXAMPLE 7 - **XUS-PEG**

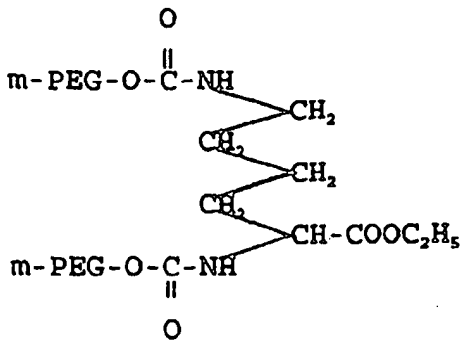
[0077]



[0078] In this example, the succinimidyl carbonate derivative of compound prepared in Example 5 was prepared according to the process described in Example 3, by reacting N-hydroxysuccinimide with the p-nitrophenyl carbonate derivative of Example 6. The recovered product yield was 84%.

EXAMPLE 8 - **U-LYS-PEG**

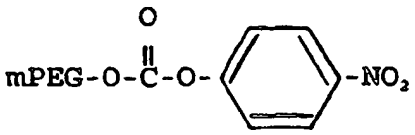
[0079]



[0080] The branched polymer depicted above was prepared by reacting mPNP — PEG with lysine ethyl ester. In particular, a mixture of 5.0 g (1.0 mmol) of the polymer, 150 mg (0.6 mmol) of lysine dihydrochloride and 140 mg (1.8 mmol) of pyridine was refluxed for 18 hours. The solvent was removed by distillation in vacuo. The residue was re-crystallized from 2-propanol to yield 4.5 g (88% yield) of product.

EXAMPLE 9 - **Synthesis of m-PNP-PEG**

[0081]



[0082] A solution of 50g (0.01 moles) of m-PEG-OH (MW=5000) in 500ml of toluene was azeotroped for 2 hrs, while removing 100ml of toluene/water. The reaction mixture was cooled to 30°C, followed by addition of 2.6 g (0.013 moles) of p-nitrophenyl chloroformate and 1.0 ml (0.013 moles) of pyridine. The resulting mixture was stirred for two hours at 45° C, followed by stirring overnight at room temperature.

[0083] The reaction mixture was then filtered through CELITE™, followed by removal of the solvent by distillation in vacuo. The residue was recrystallized from 2-propanol to yield 48.2g (93% yield) of the product.

EXAMPLES 10 and 11

[0084] Conjugates of erythropoietin (EPO) with US-PEG (Example 3) were prepared by dialyzing two 3.0 mg EPO samples (human recombinant Chinese Hamster Ovary (CHO) cell culture) into 0.1 M phosphate buffer pH 7.0 solutions using a Centricon-10 (Amicon Corporation, Beverly, MA). The first EPO solution was combined with 1.954 mg (2-fold molar excess) of the US-PEG while the second EPO solution was combined with 3.908 mg (4-fold molar excess) of the US-PEG. The reaction mixtures were stirred for one hour at room temperature (about 22-25°C). The excess polymer was removed by centrifugation and the reaction mixtures were dialyzed into 10 mM phosphate buffer, pH 8.0. Unreacted EPO was removed on an ionexchange column (2-HD column, Sepracor).

[0085] SDS-PAGE analysis confirmed that for both reaction mixtures, about two to three of the branched polymers were covalently bound to each protein molecule. The EPO activity of the conjugates was measured by colorometric assay with DA 1-K cells, a murine lymphoblastic cell line dependent on IL-3, GM-CSF and EPO for growth. The cells are grown in IMDM containing 5% FCS and incubated at 37°C in 5% CO_2 in air. The assay time is 72 hours and cell growth is monitored by MTT dye uptake. In the assay, both conjugate samples retained 40-50% of the activity of the unconjugated EPO.

EXAMPLES 12 and 13

[0086] Tumor Necrosis Factor (TNF) was conjugated with the XUS-PEG of Example 7. As a comparison, the TNF was also conjugated with the linear SC PEG, methoxypoly(ethylene glycol) succinimidyl carbonate of U.S. Patent No. 5,122,614. Both conjugates were prepared by reacting a 500 micrograms of TNF, 2.0 mg/mL, with a 25-fold molar excess of the polymer. Each reaction was carried out for 140 minutes on ice.

[0087] The ED_{50} for the branched conjugate was 0.29 ng/mL for the concentration-response curve generated by dilutions of 0.1 micrograms/mL and 0.625 ng/mL for the concentration-response curve generated by dilutions of 0.01 micrograms/mL. The ED_{50} for unmodified TNF of 0.01-0.02 ng/mL. The ED_{50} for the linear succinimidyl carbonate conjugates, ranged between 8 and 19 ng/mL.

[0088] In vitro tumoricidal and toxicity data indicated that the branched conjugate appears to be more cytotoxic than the non-branched conjugate.

[0089] While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made without departing from the spirit of the invention. It is intended to claim all such changes and modifications as fall within the true scope of the invention.

Claims

1. A branched substantially non-antigenic polymer comprising the formula:

$$(R)_nL\text{-}A$$

wherein:

(R) is a water soluble, substantially non-antigenic polymer containing a terminal C_1-C_4 group;
(n) = 2 or 3;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each (R); and
(A) is a functional group capable of bonding with a biologically active nucleophile or a moiety capable of being functionalized to react with said nucleophile.

2. The polymer of claim 1, wherein at least one (R) is a straight-chained polymer.

3. The polymer of claim 1, wherein at least one (R) is a poly(alkylene oxide).

4. The polymer of claim 3, wherein said poly(alkylene oxide) is an α-substituted poly(alkylene oxide).

5. The polymer of claim 3, wherein said poly(alkylene oxide) is selected from the group consisting of poly(ethylene glycol) homopolymers, alkyl-capped poly(ethylene oxides) and copolymers of block copolymers of poly(alkylene oxides).

6. The polymer of claim 5, wherein said poly(alkylene oxide) has a molecular weight between about 200 and about 20,000.

7. The polymer of claim 6, wherein said poly(alkylene oxide) has a molecular weight between 2,000 and about 10,000.

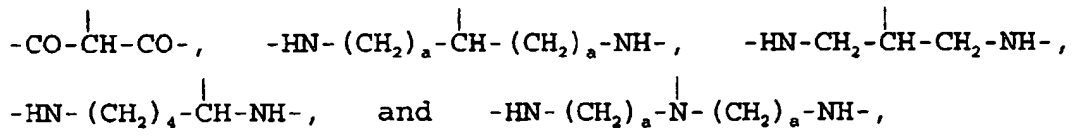
8. The polymer of claim 5, wherein said poly(ethylene glycol) homopolymer has a molecular weight of about 5,000.

9. The polymer of claim 2, wherein each (R) is a poly(ethylene glycol) homopolymer.

10. The polymer of claim 1, wherein at least one (R) is a branched polymer.

11. The activated branched polymer of claim 1, wherein (n) is two.

12. The polymer of claim 11, wherein (L) is selected from the group consisting of



wherein (a) is an integer of from 1 to 5.

13. The polymer of claim 1, wherein (A) is a functional group capable of bonding with a biologically active nucleophile selected from the group consisting of proteins, peptides, polypeptides, enzymes and chemotherapeutic molecules, or a moiety capable of being functionalized to react with said nucleophile.

14. The polymer of claim 1, wherein (A) is a moiety selected from the group consisting of hydroxyl, amino, carboxyl, carbonyl, thiol and methylene hydrogen moieties.

15. The polymer of claim 1, wherein (A) is a succinimidyl or a p-nitrophenyl carbonate active ester.

16. The polymer of claim 1, wherein (A) further comprises a spacer moiety.

17. The polymer of claim 16, wherein said spacer moiety is selected from the group consisting of alkyl groups containing up to 18 carbon atoms, cycloalkyl groups containing up to 18 carbon atoms and polymers.

18. The polymer of claim 1, wherein at least one (R) further comprises a functional group capable of covalently bonding with nucleophiles.

19. A method of forming a biologically active conjugate, comprising contacting a biologically active nucleophile with an activated branched non-antigenic polymer having a structure represented by:

$$(R)_nL\text{-}A$$

wherein:

(R) is a water soluble, substantially non-antigenic polymer containing a terminal C_1-C_4 group;
(n) = 2 or 3 ;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each (R); and
(A) is a functional group capable of forming a covalent bond with said nucleophile.

20. A polymer conjugate comprising the formula:

$$[(R)_nL\text{-}A^1]_z\text{-}(\text{nucleophile})$$

wherein:

(R) is a water soluble, substantially non-antigenic polymer containing a terminal C_1-C_4 group;
(n) = 2 or 3 ;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each (R);
(nucleophile) is a biologically active nucleophile;
(A^1) represents a covalent linkage between (L) and the biologically active nucleophile; and
(z) represents the number of polymers attached to the nucleophile.

21. The conjugate of claim 20, wherein at least one (R) is a poly(alkylene oxide).

22. The conjugate of claim 21, wherein said poly(alkylene oxide) is an α-substituted poly(alkylene oxide).

23. The conjugate of claim 21, wherein said poly(alkylene oxide) is selected from the group consisting of poly(ethylene glycol) homopolymers, alkyl-capped poly(ethylene oxides) and copolymers of block copolymers of poly(alkylene oxides).

24. The conjugate of claim 23, wherein said poly(alkylene oxide) has a molecular weight between about 200 and about 20,000.

25. The conjugate of claim 24, wherein said poly(alkylene oxide) has a molecular weight between 2,000 and about 10,000.

26. The conjugate of claim 23, wherein said poly(ethylene glycol) homopolymer has a molecular weight of about 5,000.

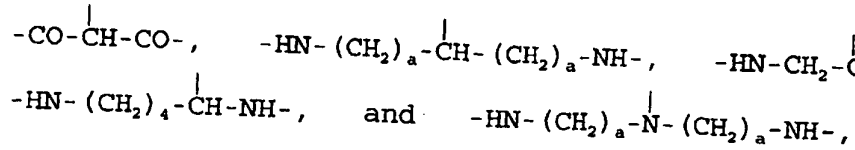
27. The conjugate of claim 21, wherein each (R) is a poly(ethylene glycol) homopolymer.

28. The conjugate of claim 20, wherein at least one (R) is a branched polymer.

29. The conjugate of claim 20, wherein (L) is a non-planar moiety.

30. The conjugate of claim 20, wherein (n) is two.

31. The conjugate of claim 30, wherein (L) is selected from the group consisting of



wherein (a) is an integer of from 1 to 5.

32. The conjugate of claim 20, wherein (A) is a functional group capable of bonding with a biologically active nucleophile selected from the group consisting of proteins, peptides, polypeptides, enzymes and chemotherapeutic molecules, or a moiety capable of being functionalized to react with said nucleophile.

33. The conjugate of claim 32, wherein said protein is selected from the group consisting of antibodies, monoclonal antibodies, fragments of antibodies and single chain-binding antigens.,

34. The conjugate of claim 20, wherein said nucleophile is a member of the group consisting of anti-neoplastics, antiinfectives, anti-anxiety agents, gastrointestinal agents, central nervous system-activating agents, analgesics, fertility agents, contraceptive agents, anti-inflammatory agents, steroidal agents, anti-urecemic agents, cardiovascular agents, vasodilating agents and vasoconstricting agents.

35. The conjugate of claim 34, wherein said antineoplastic agent is selected from the group consisting of taxol, taxanes, taxoid molecules, anthracyclines and methotrexates.

36. The branched polymer conjugate of any one of claims 20-35 for use in a method of treatment of a mammal.

37. A method of preparing a succinimidyl carbonate active ester of a branched non-antigenic polymer comprising:

i) contacting a branched non-antigenic polymer having a structure represented by:

$$(R)_n L\text{-}A$$

wherein:

(R) independently comprises a water-soluble substantially non-antigenic polymer;
(n) = 2 or 3;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each non-antigenic polymer; and
(A) is a functional group capable of forming a covalent bond with a nucleophile;

with p-nitrophenyl chloroformate; and

ii) reacting the p-nitrophenyl carbonate active ester of step i) with N-hydroxysuccinimide.

38. The method of claim 37, wherein (A) comprises a spacer moiety proximal to said aliphatic linking group (L).

39. A method of preparing a succinimidyl carbonate active ester of a branched non-antigenic polymer comprising contacting a branched non-antigenic polymer having a structure represented by:

$$(R)_nL\text{-}A$$

wherein:

(R) independently comprises a water-soluble substantially non-antigenic polymer;
(n) = 2 or 3;
(L) is an aliphatic linking moiety selected from the group consisting of substituted alkyl diamines and triamines, lysine esters and malonic ester derivatives covalently linked to each non-antigenic polymer; and
(A) is a functional group capable of forming a covalent bond with a nucleophile;

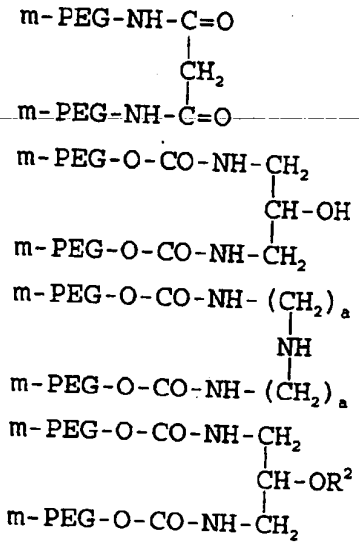
with bis-succinimidyl carbonate.

40. The method of claim 39, wherein (A) further comprises a spacer moiety.

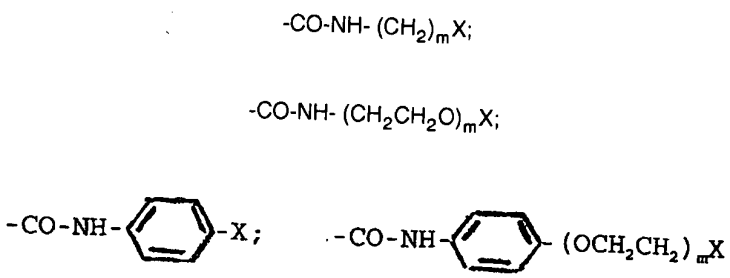
41. The polymer of claim 1, wherein said C_1-C_4 terminal group is methoxy.

42. The polymer of claim 1, wherein said (A) is selected from the group consisting of carbonyl imidazole, azlactones, cyclic imide thiones, isocyanates, isothiocyanates, primary amines, hydrazines, acyl hydrazines, carbazates, semicarbazates, thiocarbazates and phenylglyoxals.

43. The polymer of claim 1 comprising a structure selected from the group consisting of

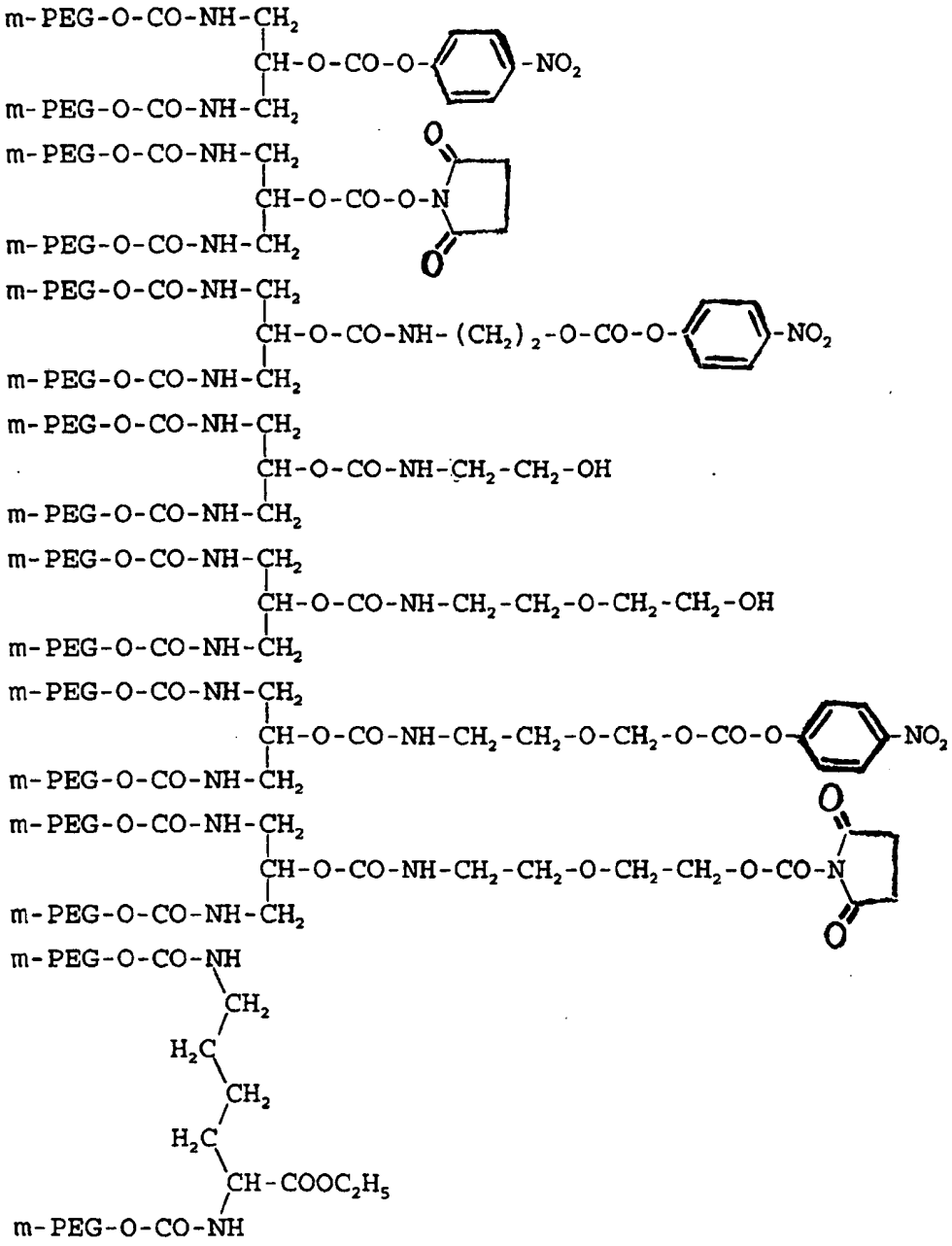


wherein (a) is 1 to 5 and R^2 is a spacer moiety selected from the group consisting of

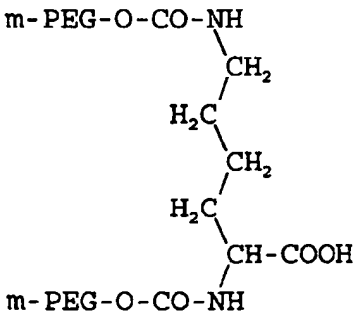


wherein (m) is an integer between 1 and 18 inclusive and (X) is H, OH, NH_2 or COOH.

44. The polymer of claim 1 comprising a structure selected from the group consisting of



and



45. The polymer of claim 1, wherein said (R) is linked to said (L) by a linkage selected from the group consisting of urethane, amide, ether, amine, urea, thio and thiol.

46. The polymer conjugate of claim 20, wherein said nucleophile is selected from the group consisting of α-, β- and γ-interferons.

47. The polymer conjugate of claim 46, wherein said interferon is an α-interferon.

Patentansprüche

1. Verzweigtes, im wesentlichen nichtantigenes Polymer der Formel

$$(R)_nL\text{-}A,$$

wobei

(R) ein wasserlösliches, im wesentlichen nichtantigenes Polymer ist, das eine terminale C_1-C_4-Gruppe enthält;

(n) = 2 oder 3 ist;

(L) eine aliphatische Verknüpfungsgruppe ist, die aus der Gruppe ausgewählt ist, die aus substituierten Alkyldiaminen und -triaminen, Lysinestern und Malonsäureesterderivaten, die an jedes (R) kovalent gebunden sind, besteht; und

(A) eine funktionelle Gruppe, die in der Lage ist, an ein biologisch aktives Nucleophil zu binden, oder eine Struktureinheit, die so funktionalisiert werden kann, dass sie mit dem Nucleophil reagiert, ist.

2. Polymer gemäß Anspruch 1, wobei wenigstens ein (R) ein geradkettiges Polymer ist.

3. Polymer gemäß Anspruch 1, wobei wenigstens ein (R) ein Polyalkylenoxid ist.

4. Polymer gemäß Anspruch 3, wobei das Polyalkylenoxid ein α-substituiertes Polyalkylenoxid ist.

5. Polymer gemäß Anspruch 3, wobei das Polyalkylenoxid aus der Gruppe ausgewählt ist, die aus Polyethylenglycol-Homopolymeren, alkylverkappten Polyethylenoxiden und Copolymeren von Blockcopolymeren von Polyalkylenoxiden besteht.

6. Polymer gemäß Anspruch 5, wobei das Polyalkylenoxid ein Molekulargewicht zwischen etwa 200 und etwa 20 000 hat.

7. Polymer gemäß Anspruch 6, wobei das Polyalkylenoxid ein Molekulargewicht zwischen 2000 und etwa 10 000 hat.

8. Polymer gemäß Anspruch 5, wobei das Polyethylenglycol-Homopolymer ein Molekulargewicht von etwa 5000 hat.

9. Polymer gemäß Anspruch 2, wobei jedes (R) ein Polyethylenglycol-Homopolymer ist.

10. Polymer gemäß Anspruch 1, wobei wenigstens ein (R) ein verzweigtes Polymer ist.

11. Aktiviertes verzweigtes Polymer gemäß Anspruch 1, wobei (n) gleich 2 ist.

12. Polymer gemäß Anspruch 11, wobei (L) aus der Gruppe ausgewählt ist, die aus

$$-CO-\underset{|}{CH}-CO-, \quad -HN-(CH_2)_a-\underset{|}{CH}-(CH_2)_a-NH-, \quad -HN-CH_2-\underset{|}{CH}-CH_2-NH-,$$

$$-HN-(CH_2)_4-\underset{|}{CH}-NH- \text{ und } -HN-(CH_2)_a-\underset{|}{N}-(CH_2)_a-NH-$$

besteht, wobei (a) eine ganze Zahl von 1 bis 5 ist.

13. Polymer gemäß Anspruch 1, wobei (A) eine funktionelle Gruppe, die in der Lage ist, an ein biologisch aktives Nucleophil zu binden, das aus der Gruppe ausgewählt ist, die aus Proteinen, Peptiden, Polypeptiden, Enzymen und chemotherapeutischen Molekülen besteht, oder eine Struktureinheit, die so funktionalisiert werden kann, dass sie mit dem Nucleophil reagiert, ist.

14. Polymer gemäß Anspruch 1, wobei (A) eine Struktureinheit ist, die aus der Gruppe ausgewählt ist, die aus Hydroxy, Amino, Carboxy, Carbonyl, Thiol und Struktureinheiten mit Methylenwasserstoff besteht.

15. Polymer gemäß Anspruch 1, wobei (A) ein aktiver Succinimidylkohlensäureester oder ein aktiver p-Nitrophenylkohlensäureester ist.

16. Polymer gemäß Anspruch 1, wobei (A) weiterhin eine Spacergruppe umfasst.

17. Polymer gemäß Anspruch 16, wobei die Spacergruppe aus der Gruppe ausgewählt ist, die aus Alkylgruppen mit bis zu 18 Kohlenstoffatomen, Cycloalkylgruppen mit bis zu 18 Kohlenstoffatomen und Polymeren besteht.

18. Polymer gemäß Anspruch 1, wobei wenigstens ein (R) weiterhin eine funktionelle Gruppe umfasst, die in der Lage ist, kovalent an Nucleophile zu binden.

19. Verfahren zur Bildung eines biologisch aktiven Konjugats, umfassend das In-Kontakt-Bringen eines biologisch aktiven Nucleophils mit einem aktivierten verzweigten nichtantigenen Polymer mit einer Struktur, die durch

$$(R)_nL\text{-}A$$

dargestellt wird, wobei

(R) ein wasserlösliches, im wesentlichen nichtantigenes Polymer ist, das eine terminale C_1-C_4-Gruppe enthält;

(n) = 2 oder 3 ist;

(L) eine aliphatische Verknüpfungsgruppe ist, die aus der Gruppe ausgewählt ist, die aus substituierten Alkyldiaminen und -triaminen, Lysinestern und Malonsäureesterderivaten, die an jedes (R) kovalent gebunden sind, besteht; und

(A) eine funktionelle Gruppe ist, die in der Lage ist, eine kovalente Bindung mit dem Nucleophil zu bilden.

20. Polymerkonjugat der Formel

$$[(R)_nL\text{-}A^1]_z\text{-(Nucleophil)},$$

wobei

(R) ein wasserlösliches, im wesentlichen nichtantigenes Polymer ist, das eine terminale C_1-C_4-Gruppe enthält;

(n) = 2 oder 3 ist;

(L) eine aliphatische Verknüpfungsgruppe ist, die aus der Gruppe ausgewählt ist, die aus substituierten Alkyldiaminen und -triaminen, Lysinestern und Malonsäureesterderivaten, die an jedes (R) kovalent gebunden sind, besteht;

(Nucleophil) ein biologisch aktives Nucleophil ist;

(A^1) eine kovalente Verknüpfung zwischen (L) und dem biologisch aktiven Nucleophil darstellt; und

(z) die Anzahl der an das Nucleophil gebundenen Polymere darstellt.

21. Konjugat gemäß Anspruch 20, wobei wenigstens ein (R) ein Polyalkylenoxid ist.

22. Konjugat gemäß Anspruch 21, wobei das Polyalkylenoxid ein α-substituiertes Polyalkylenoxid ist.

23. Konjugat gemäß Anspruch 21, wobei das Polyalkylenoxid aus der Gruppe ausgewählt ist, die aus Polyethylenglycol-Homopolymeren, alkylverkappten Polyethylenoxiden und Copolymeren von Blockcopolymeren von Polyalkylenoxiden besteht.

24. Konjugat gemäß Anspruch 23, wobei das Polyalkylenoxid ein Molekulargewicht zwischen etwa 200 und etwa 20 000 hat.

25. Konjugat gemäß Anspruch 24, wobei das Polyalkylenoxid ein Molekulargewicht zwischen 2000 und etwa 10 000 hat.

26. Konjugat gemäß Anspruch 23, wobei das Polyethylenglycol-Homopolymer ein Molekulargewicht von etwa 5000 hat.

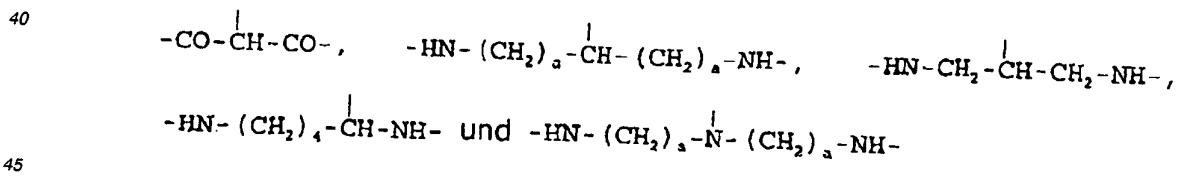
27. Konjugat gemäß Anspruch 21, wobei jedes (R) ein Polyethylenglycol-Homopolymer ist.

28. Konjugat gemäß Anspruch 20, wobei wenigstens ein (R) ein verzweigtes Polymer ist.

29. Konjugat gemäß Anspruch 20, wobei (L) eine nichtplanare Struktureinheit ist.

30. Konjugat gemäß Anspruch 20, wobei (n) gleich 2 ist.

31. Konjugat gemäß Anspruch 30, wobei (L) aus der Gruppe ausgewählt ist, die aus



besteht, wobei (a) eine ganze Zahl von 1 bis 5 ist.

32. Konjugat gemäß Anspruch 20, wobei (A) eine funktionelle Gruppe, die in der Lage ist, an ein biologisch aktives Nucleophil zu binden, das aus der Gruppe ausgewählt ist, die aus Proteinen, Peptiden, Polypeptiden, Enzymen und chemotherapeutischen Molekülen besteht, oder eine Struktureinheit, die so funktionalisiert werden kann, dass sie mit dem Nucleophil reagiert, ist.

33. Konjugat gemäß Anspruch 32, wobei das Protein aus der Gruppe ausgewählt ist, die aus Antikörpern, monoklonalen Antikörpern, Fragmenten von Antikörpern und einzelkettenbindenden Antigenen besteht.

34. Konjugat gemäß Anspruch 20, wobei das Nucleophil ein Vertreter der Gruppe ist, die aus Antineoplastika, Antiinfektiva, angstlösenden Mitteln, gastrointestinalen Mitteln, Zentralnervensystem-aktivierenden Mitteln, Analgetika, Fruchtbarkeitsmitteln, Kontrazeptiva, entzündungshemmenden Mitteln, steroidalen Mitteln, antiurikämischen Mit-

teln, cardiovaskulären Mitteln, gefäßerweiternden Mitteln und gefäßverengenden Mitteln besteht.

35. Konjugat gemäß Anspruch 34, wobei das Antineoplastikum aus der Gruppe ausgewählt ist, die aus Taxol, Taxanen, Taxoidmolekülen, Anthracyclinen und Methotrexaten besteht.

36. Verzweigtes Polymerkonjugat gemäß einem der Ansprüche 20-35 zur Verwendung in einem Verfahren zur Behandlung eines Säugers.

37. Verfahren zur Herstellung eines aktiven Succinimidylkohlensäureesters eines verzweigten nichtantigenen Polymers, umfassend:

i) In-Kontakt-Bringen eines verzweigten nichtantigenen Polymers mit einer Struktur, die durch

$$(R)_nL\text{-}A$$

dargestellt wird, wobei

(R) unabhängig ein wasserlösliches, im wesentlichen nichtantigenes Polymer umfasst;

(n) = 2 oder 3 ist;

(L) eine aliphatische Verknüpfungsgruppe ist, die aus der Gruppe ausgewählt ist, die aus substituierten Alkyldiaminen und -triaminen, Lysinestern und Malonsäureesterderivaten, die an jedes (R) kovalent gebunden sind, besteht; und

(A) eine funktionelle Gruppe ist, die in der Lage ist, eine kovalente Bindung mit einem Nucleophil zu bilden;

mit p-Nitrophenylchlorformiat; und

ii) Umsetzen des aktiven p-Nitrophenylkohlensäureesters von Schritt i) mit N-Hydroxysuccinimid.

38. Verfahren gemäß Anspruch 37, wobei (A) eine Spacergruppe proximal zu der aliphatischen Verknüpfungsgruppe (L) umfasst.

39. Verfahren zur Herstellung eines aktiven Succinimidylkohlensäureesters eines verzweigten nichtantigenen Polymers, umfassend das In-Kontakt-Bringen eines verzweigten nichtantigenen Polymers mit einer Struktur, die durch

$$(R)_nL\text{-}A$$

dargestellt wird, wobei

(R) unabhängig ein wasserlösliches, im wesentlichen nichtantigenes Polymer umfasst;

(n) = 2 oder 3 ist;

(L) eine aliphatische Verknüpfungsgruppe ist, die aus der Gruppe ausgewählt ist, die aus substituierten Alkyldiaminen und -triaminen, Lysinestern und Malonsäureesterderivaten, die an jedes (R) kovalent gebunden sind, besteht; und

(A) eine funktionelle Gruppe ist, die in der Lage ist, eine kovalente Bindung mit einem Nucleophil zu bilden;

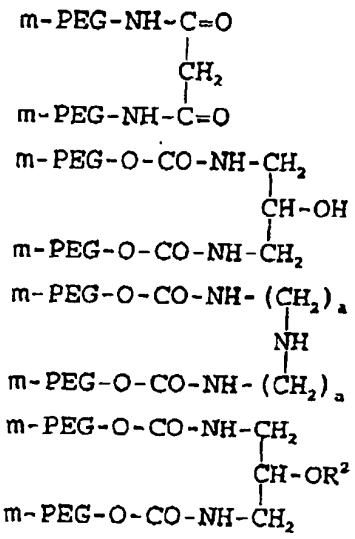
mit Bis(succinimidyl)carbonat.

40. Verfahren gemäß Anspruch 39, wobei (A) weiterhin eine Spacergruppe umfasst.

41. Polymer gemäß Anspruch 1, wobei es sich bei der terminalen C_1-C_4-Gruppe um Methoxy handelt.

42. Polymer gemäß Anspruch 1, wobei (A) aus der Gruppe ausgewählt ist, die aus Carbonylimidazol, Azlactonen, cyclischen Imidthionen, Isocyanaten, Isothiocyanaten, primären Aminen, Hydrazinen, Acylhydrazinen, Carbazaten, Semicarbazaten, Thiocarbazaten und Phenylglyoxalen besteht.

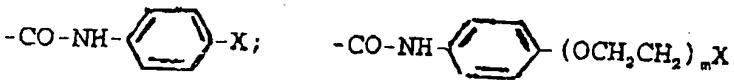
43. Polymer gemäß Anspruch 1, das eine Struktur umfasst, die aus der Gruppe ausgewählt ist, die aus folgenden besteht:



wobei (a) 1 bis 5 beträgt und R^2 eine Spacergruppe ist, die aus der Gruppe ausgewählt ist, die aus folgenden besteht:

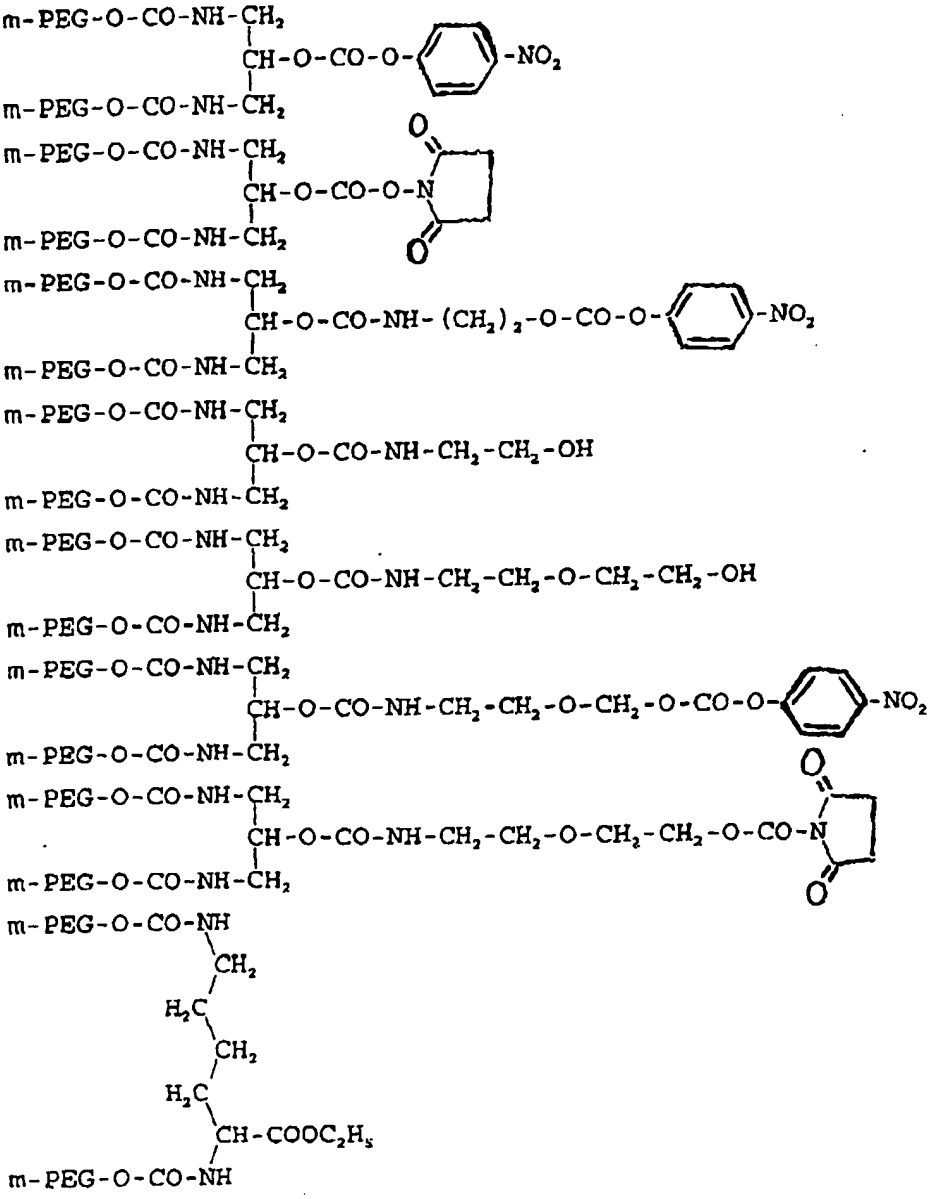
$$-CO-NH-(CH_2)_mX;$$

$$-CO-NH-(CH_2CH_2O)_mX;$$

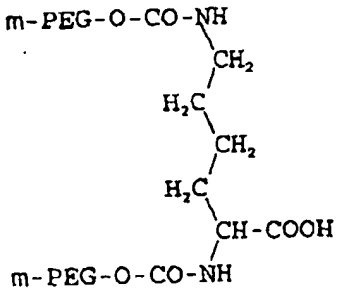


wobei (m) eine ganze Zahl zwischen 1 und 18 inklusive ist und (X) H, OH, NH_2 oder COOH ist.

44. Polymer gemäß Anspruch 1, das eine Struktur umfasst, die aus der Gruppe ausgewählt ist, die aus folgenden besteht:



und



45. Polymer gemäß Anspruch 1, wobei das (R) über eine Verknüpfung mit dem (L) verknüpft ist, die aus der Gruppe

ausgewählt ist, die aus Urethan, Amid, Ether, Amin, Harnstoff, Thio und Thiol besteht.

46. Polymerkonjugat gemäß Anspruch 20, wobei das Nucleophil aus der Gruppe ausgewählt ist, die aus α-, β- und γ-Interferon besteht.

47. Polymerkonjugat gemäß Anspruch 46, wobei es sich bei dem Interferon um α-Interferon handelt.

Revendications

1. Polymère ramifié sensiblement non antigénique comprenant la formule :

$$(R)_nL\text{-}A$$

où :

(R) est un polymère hydrosoluble sensiblement non antigénique contenant un groupe terminal en C_1-C_4 ;
(n) = 2 ou 3 ;
(L) est une entité de liaison aliphatique choisie dans le groupe consistant en les alkyldiamines et triamines substituées, les esters de lysine et les dérivés d'esters maloniques liés de manière covalente à chaque (R) ; et
(A) est un groupe fonctionnel capable de se lier à un nucléophile biologiquement actif ou une entité capable d'être fonctionnalisée pour réagir avec ledit nucléophile.

2. Polymère selon la revendication 1, où au moins un (R) est un polymère linéaire.

3. Polymère selon la revendication 1, où au moins un (R) est un poly(oxyde d'alkylène).

4. Polymère selon la revendication 3, où ledit poly(oxyde d'alkylène) est un poly(oxyde d'alkylène) α-substitué.

5. Polymère selon la revendication 3, où ledit poly(oxyde d'alkylène) est choisi dans le groupe consistant en les homopolymères de poly(éthylène-glycol), les poly(oxydes d'éthylène) à terminaisons alkyle et les copolymères de copolymères séquencés de poly(oxydes d'alkylène).

6. Polymère selon la revendication 5, où ledit poly(oxyde d'alkylène) a une masse moléculaire située entre environ 200 et environ 20 000.

7. Polymère selon la revendication 6, où ledit poly(oxyde d'alkylène) a une masse moléculaire située entre 2 000 et environ 10 000.

8. Polymère selon la revendication 5, où ledit homopolymère de poly(éthylèneglycol) a une masse moléculaire d'environ 5 000.

9. Polymère selon la revendication 2, où chaque (R) est un homopolymère de poly(éthylèneglycol).

10. Polymère selon la revendication 1, où au moins un (R) est un polymère ramifié.

11. Polymère ramifié activé selon la revendication 1, où (n) est deux.

12. Polymère selon la revendication 11, où (L) est choisi dans le groupe consistant en

$$-CO\text{-}\underset{|}{C}H\text{-}CO-,\ -HN\text{-}(CH_2)_a\text{-}\underset{|}{C}H\text{-}(CH_2)_a\text{-}NH-,\ -HN\text{-}CH_2\text{-}\underset{|}{C}H\text{-}CH_2\text{-}NH-,$$

$$-HN\text{-}(CH_2)_4\text{-}\underset{|}{C}H\text{-}NH-,\ \text{et}\ -HN\text{-}(CH_2)_a\text{-}\underset{|}{N}\text{-}(CH_2)_a\text{-}NH-,$$

où (a) est un entier de 1 à 5.

13. Polymère selon la revendication 1, où (A) est un groupe fonctionnel capable de se lier à un nucléophile biologiquement actif choisi dans le groupe consistant en les protéines, les peptides, les polypeptides, les enzymes et les molécules chimiothérapeutiques, ou une entité capable d'être fonctionnalisée pour réagir avec ledit nucléophile.

14. Polymère selon la revendication 1, où (A) est une entité choisie dans le groupe consistant en les entités hydroxyle, amino, carboxyle, carbonyle, thiole et hydrogène de méthylène.

15. Polymère selon la revendication 1, où (A) est un ester actif carbonate de succinimidyle ou de p-nitrophényle.

16. Polymère selon la revendication 1, où (A) comprend en outre une entité espaceur.

17. Polymère selon la revendication 16, où ladite entité espaceur est choisie dans le groupe consistant en les groupes alkyle contenant jusqu'à 18 atomes de carbone, les groupes cycloalkyle contenant jusqu'à 18 atomes de carbone et les polymères.

18. Polymère selon la revendication 1, ou au moins un (R) comprend en outre un groupe fonctionnel capable de se lier de manière covalente à des nucléophiles.

19. Procédé de formation d'un conjugué biologiquement actif comprenant la mise en contact d'un nucléophile biologiquement actif avec un polymère ramifié activé non antigénique ayant une structure représentée par :

$$(R)_nL\text{-}A$$

où:

(R) est un polymère hydrosoluble sensiblement non antigénique contenant un groupe terminal en C_1-C_4 ;
(n) = 2 ou 3 ;
(L) est une entité de liaison aliphatique choisie dans le groupe consistant en les alkyldiamines et triamines substituées, les esters de lysine et les dérivés d'esters maloniques liés de manière covalente à chaque (R) ; et
(A) est un groupe fonctionnel capable de former une liaison covalente avec ledit nucléophile.

20. Conjugué de polymères comprenant la formule :

$$[(R)_nL\text{-}A^1]_z - (\text{nucléophile})$$

où:

(R) est un polymère hydrosoluble sensiblement non antigénique contenant un groupe terminal en C_1-C_4 ;
(n) = 2 ou 3 ;
(L) est une entité de liaison aliphatique choisie dans le groupe consistant en les alkyldiamines et triamines substituées, les esters de lysine et les dérivés d'esters maloniques liés de manière covalente à chaque (R) ;
(nucléophile) est un nucléophile biologiquement actif ;
(A^1) représente une liaison covalente entre (L) et le nucléophile biologiquement actif ; et
(z) représente le nombre de polymères fixés au nculéophile.

21. Conjugué selon la revendication 20, où au moins un (R) est un poly(oxyde d'alkylène).

22. Conjugué selon la revendication 21, où ledit poly(oxyde d'alkylène) est un poly(oxyde d'alkylène) α-substitué.

23. Conjugué selon la revendication 21, où ledit poly(oxyde d'alkylène) est choisi dans le groupe consistant en les homopolymères de poly(éthylèneglycol), les poly(oxydes d'éthylène) à terminaisons alkyle et les copolymères de copolymères séquencés de poly(oxydes d'alkylène)

24. Conjugué selon la revendication 23, où ledit poly(oxyde d'alkylène) a une masse moléculaire située entre environ

200 et environ 20 000.

25. Conjugué selon la revendication 24, où ledit poly(oxyde d'alkylène) a une masse moléculaire située entre 2 000 et environ 10 000.

26. Conjugué selon la revendication 23, où ledit homopolymère de poly(éthylèneglycol) a une masse moléculaire d'environ 5 000.

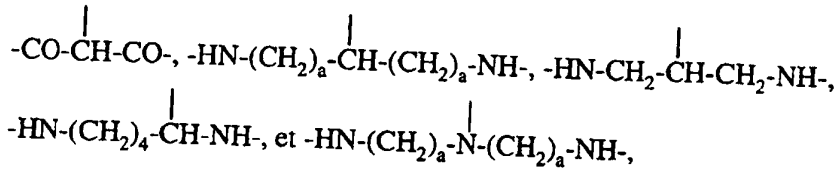
27. Conjugué selon la revendication 21, où chaque (R) est un homopolymère de poly(éthylèneglycol).

28. Conjugué selon la revendication 20, où au moins un (R) est un polymère ramifié.

29. Conjugué selon la revendication 20, où (L) est une entité non plane.

30. Conjugué selon la revendication 20, où (n) est deux.

31. Conjugué selon la revendication 30, où (L) est choisi dans le groupe consistant en



où (a) est un entier de 1 à 5.

32. Conjugué selon la revendication 20, où (A) est un groupe fonctionnel capable de se lier à un nucléophile biologiquement actif choisi dans le groupe consistant en les protéines, les peptides, les polypeptides, les enzymes et les molécules chimiothérapeutiques, ou une entité capable d'être fonctionnalisée pour réagir avec ledit nucléophile.

33. Conjugué selon la revendication 32, où ladite protéine est choisie dans le groupe consistant en les anticorps, les anticorps monoclonaux, les fragments d'anticorps et les antigènes de liaison à une seule chaîne.

34. Conjugué selon la revendication 20, où ledit nucléophile est un membre du groupe consistant en les anti-néoplasiques, les anti-infectieux, les agents anti-angoisse, les agents gastrointestinaux, les agents activant le système nerveux central, les analgésiques, les agents de fécondité, les agents contraceptifs, les agents anti-inflammatoires, les agents stéroïdiens, les agents anti-urécémiques, les agents cardiovasculaires, les agents vasodilatateurs et les agents vasoconstricteurs.

35. Conjugué selon la revendication 34, où ledit agent antinéoplasique est choisi dans le groupe consistant en le taxol, les taxanes, les molécules taxoïdes, les anthracyclines et les méthothrexates.

36. Conjugué de polymères ramifiés selon l'une quelconque des revendications 20-35, destiné à être utilisé dans un procédé de traitement d'un mammifère.

37. Procédé de préparation d'un ester actif carbonate de succinimidyle d'un polymère non antigénique ramifié comprenant :

i) la mise en contact d'un polymère non antigénique ramifié ayant une structure représentée par :

$$(R)_nL\text{-}A$$

où :

(R) comprend indépendamment un polymère hydrosoluble sensiblement non antigénique ;

(n) = 2 ou 3 ;
(L) est une entité de liaison aliphatique choisie dans le groupe consistant en les alkyldiamines et triamines substituées, les éthers de lysine et les dérivés d'esters maloniques liés de manière covalente à chaque polymère non antigénique; et
(A) est un groupe fonctionnel capable de former une liaison covalente avec un nucléophile;

avec le chloroformiate de p-nitrophényle ; et

ii) la réaction de l'ester actif carbonate de p-nitrophényle de l'étape i) avec le N-hydroxysuccinimide.

38. Procédé selon la revendication 37, où (A) comprend une entité espaceur à proximité dudit groupe de liaison aliphatique (L).

39. Procédé de préparation d'un ester actif carbonate de succinimidyle d'un polymère non antigénique ramifié comprenant la mise en contact d'un polymère non antigénique ramifié ayant une structure représentée par :

$$(R)_nL\text{-}A$$

où :

(R) comprend indépendamment un polymère hydrosoluble sensiblement non antigénique ;
(n) = 2 ou 3 ;
(L) est une entité de liaison aliphatique choisie dans le groupe consistant en les alkyldiamines et triamines substituées, les esters de lysine et les dérivés d'esters maloniques liés de manière covalente à chaque polymère non antigénique ; et
(A) est un groupe fonctionnel capable de former une liaison covalente avec un nucléophile ;

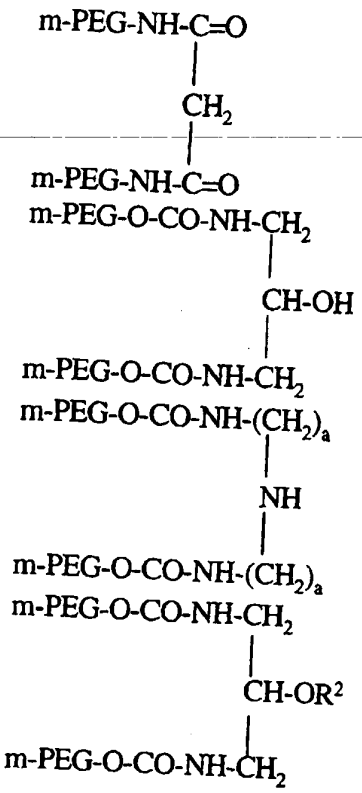
avec le carbonate de bis-succinimidyle.

40. Procédé selon la revendication 39, où (A) comprend en outre une entité espaceur.

41. Polymère selon la revendication 1, où ledit groupe terminal en C_1-C_4 est méthoxy.

42. Polymère selon la revendication 1, où ledit (A) est choisi dans le groupe consistant en le carbonylimidazole, les azlactones, les imidothiones cycliques, les isocyanates, les isothiocyanates, les amines primaires, les hydrazines, les acylhydrazines, les carbazates, les semicarbazates, les thiocarbazates et les phénylglyoxals.

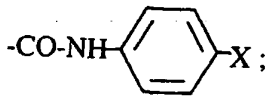
43. Polymère selon la revendication 1, comprenant une structure choisie dans le groupe consistant en

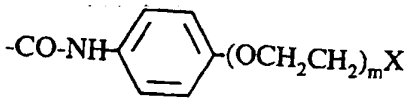


où (a) est 1 à 5 et R^2 est une entité espaceur choisie dans le groupe consistant en

$$-CO-NH-(CH_2)_mX\ ;$$

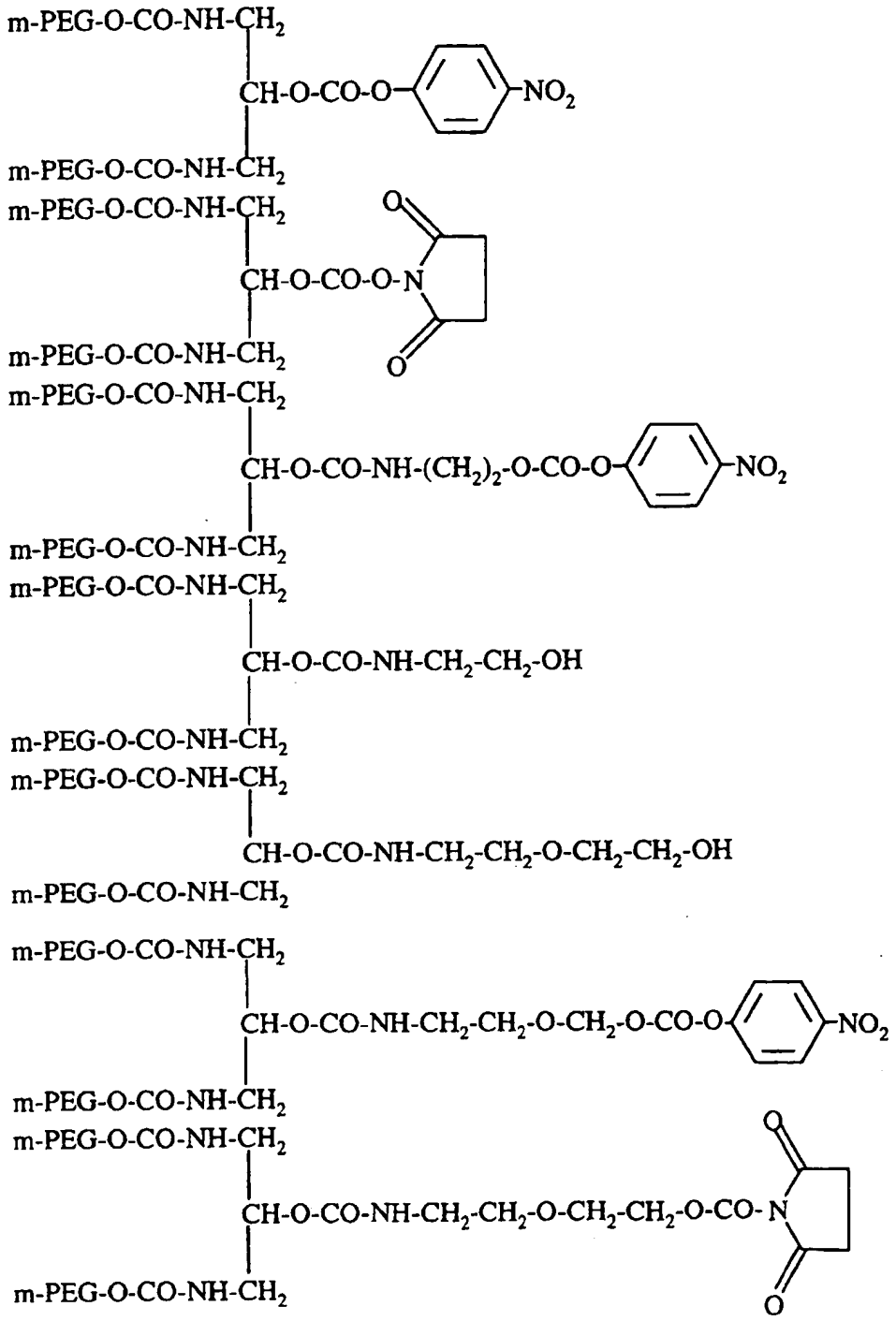
$$-CO-NH-(CH_2CH_2O)_mX\ ;$$

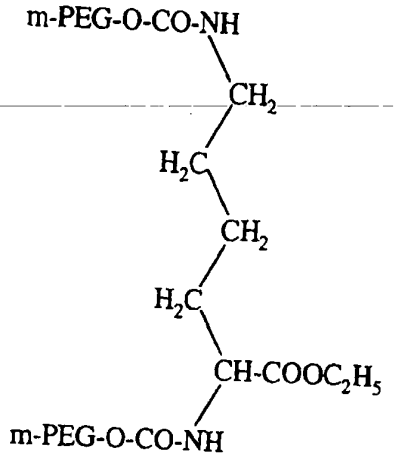




où (m) est un entier de 1 à 18 inclus et (X) est H, OH, NH_2 ou COOH.

44. Polymère selon la revendication 1, comprenant une structure choisie dans le groupe consistant en





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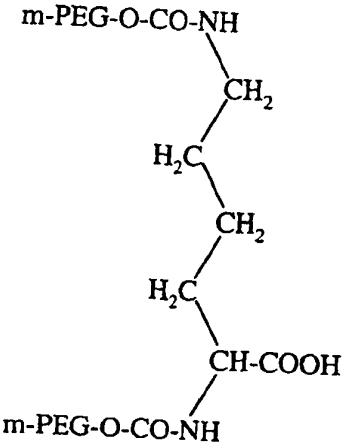

45. Polymère selon la revendication 1, où ledit (R) est lié audit (L) par une liaison choisie dans le groupe consistant en uréthane, amide, éther, amine, urée, thio et thiol.

46. Conjugué de polymères selon la revendication 20, où ledit nucléophile est choisi dans le groupe consistant en les interférons α, β et γ.

47. Conjugué de polymères selon la revendication 46, où ledit interféron est un interféron α.